Presence of the minor *EGFR* T790M mutation is associated with drug-sensitive *EGFR* mutations in lung adenocarcinoma patients

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Abstract. The T790M mutation in the epidermal growth factor receptor (*EGFR*) gene is known to be associated with the acquired resistance of lung adenocarcinoma patients to EGFR-tyrosine kinase inhibitors (EGFR-TKIs). The minor T790M mutant allele is occasionally detected in EGFR-TKI-naive tumor samples, yet findings concerning the clinical impact of the minor T790M mutation vary among previous studies. In the present study, we assessed the clinical impact of the minor T790M mutation using a novel, highly sensitive assay combining high-resolution melting (HRM), mutant-enriched PCR and co-amplification at a lower denaturation temperature (COLD)-PCR. We determined the T790M mutational status in 146 surgically resected lung adenocarcinomas without a history of EGFR-TKI treatment using mutant-enriched COLD-HRM (MEC-HRM) and standard HRM assays. The sensitivities of the MEC-HRM and standard HRM assays for the detection of T790M-mutant alleles among wild-type alleles were 0.01 and 10%, respectively. Although the T790M mutation was not detected using a standard HRM assay, we identified 19 (13%) T790M mutations using the MEC-HRM assay and defined these 19 mutations as minor T790M mutations. The proportion of T790M alleles was <0.1% in 17 (84%) of the 19 samples. Multivariate analyses revealed that a minor T790M mutation was significantly associated with the presence of *EGFR* exon 19 deletions or the L858R mutation (both of which are drug-sensitive *EGFR* mutations) (P=0.04). In conclusion, the minor *EGFR* T790M mutations were present in 13% of EGFR-TKI-naive surgically resected lung adenocarcinomas and were associated with drug-sensitive *EGFR* mutations.

Introduction

Activating mutations of the epidermal growth factor receptor (*EGFR*) gene are characteristic genetic alterations in non-small lung cancer (NSCLC) patients, particularly in those with lung adenocarcinomas (1-3). *EGFR* exon 19 deletions or the exon 21 L858R mutation account for more than 90% of all *EGFR* mutations (4,5). Of particular importance, these mutations are known as predictors of a favorable clinical outcome in response to treatment with EGFR-tyrosine kinase inhibitors (EGFR-TKIs) (1,2). Although ~80% of NSCLC patients with drug-sensitive *EGFR* mutations such as *EGFR* exon 19 deletions or the exon 21 L858R mutation initially show satisfactory responsiveness to EGFR-TKI treatment (1,2), acquired resistance to EGFR-TKIs occurs in most cases (6). Half of all resistance to EGFR-TKIs is caused by an acquired T790M mutation in exon 20 of the *EGFR* gene (7-9). The T790M mutation has been reported to cause EGFR-TKI resistance by sterically hindering the binding site of gefitinib and erlotinib, two first-generation EGFR-TKIs (7), thereby causing a relative decrease in binding with EGFR-TKIs (10).

The T790M mutation has been detected in some patients who have not been treated with EGFR-TKIs (11-16). Since the incidence of a T790M mutation has been reported to range from 0.02 to 0.05% in all surgically resected NSCLC patients based on studies using direct sequencing (2,17), it is difficult to clarify the association due to the T790M mutation and clinico-pathological factors because of the limitation in the number of cases. Using highly sensitive assays, the clinical impact of the minor T790M mutation in NSCLC patients with EGFR-TKI treatment has been previously investigated (11,14,15). However, the impact on EGFR-TKI-naive NSCLC patients has not been adequately investigated (12,13,16). Recently, various therapies to overcome the T790M mutation are being developed. These include next generation EGFR-TKIs (18) and combination

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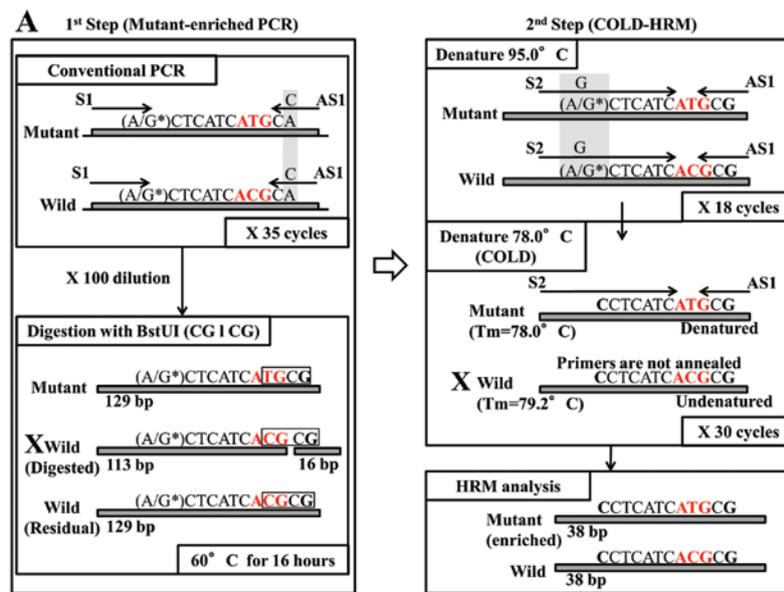E-mail: toyooka@md.okayama-u.ac.jp

Figure 1. (A) Principle of the mutant-enriched COLD-HRM (MEC-HRM) assay. In the first step (left box), the upper box indicates the conventional PCR step for the amplification of *EGFR* exon 20, including codon 790. The mismatched site of the AS1 primer to introduce the CGCG sequence (framed) is emphasized with light gray shading. The exchanged bases are emphasized with bold print. An asterisk indicates the SNP site. The lower box indicates the step for the selective digestion of the wild-type amplicon. The crosses are applied on reduced amplicons. After digestion, the product that includes the mutant amplicon and residual undigested wild-type amplicon is used for the second step. The upper box indicates the first step of the real-time PCR for the amplification of the template product. The mismatched site of the S2 primer to diminish the influence of SNP is emphasized in light gray shading. The lower box indicates the second step, which is a COLD-PCR step for the selective amplification of the mutant. The Td (78.0°C) was lower than Tm of the wild-type amplicon; thus, they were not denatured and the primers were not annealed. The final products were analyzed using an HRM analysis.

therapies (19). Minor T790M mutated clones are enriched by EGFR-TKI treatment (12,16); thus, early detection of a minor T790M mutation using a highly sensitive assay may be useful for predicting the cause of resistance to EGFR-TKIs and for selecting optimum therapeutic strategies.

In the present study, we determined the T790M mutational status using high-resolution melting (HRM) analysis combined with mutant-enriched (12,20) and co-amplification at lower denaturation temperature (COLD)-polymerase chain reactions (PCRs) (21-24) to investigate the relationship between the presence of minor T790M mutations and clinicopathological factors in EGFR-TKI-naive lung adenocarcinoma patients with pulmonary resection.

Materials and methods

Clinical samples, cell lines and DNA extraction. We studied 146 patients with lung adenocarcinomas who underwent surgical resection without a preoperative history of EGFR-TKI treatment at our institute between 2006 and 2009. Approval of the Institutional Review Board and the informed consent of each patient were obtained. After pulmonary resection, fresh tissue was immediately frozen and stored at -80°C. DNA was extracted from the frozen tissue using proteinase K treatment followed by phenol-chloroform extraction (25). We also used a human bronchial epithelial cell line (HBEC-5KT) harboring the wild-type *EGFR* gene and the NCI-H1975 cell line (H1975) harboring the *EGFR* mutations L858R and T790M as negative and positive controls, respectively. These cell lines were kindly provided by Dr Adi F. Gazdar (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX,

USA). DNA of the cell lines was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

Plasmids containing exon 20 of the EGFR gene. We used a plasmid containing *EGFR* exon 20 with the T790M mutation, which is one of the standardized plasmids containing each of the *EGFR* mutations occurring in the exons, and was used in a study by Goto *et al* (26). We also constructed a plasmid containing wild-type *EGFR* exon 20 using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) as previously reported by us (27). The sequences of each plasmid were confirmed by direct sequencing.

Detection of EGFR exon 19 deletions and the L858R mutation. *EGFR* exon 19 deletions and the exon 21 L858R mutation were examined using a restriction fragment length polymorphism (RFLP) assay without the enrichment of mutant alleles, as previously reported by us (20). We defined these *EGFR* exon 19 deletions and the exon 21 L858R mutation as drug-sensitive *EGFR* mutations.

Detection of the EGFR T790M mutation. Mutant-enriched COLD-HRM (MEC-HRM) is a two-step PCR-based assay combining a standard HRM assay with a mutant-enriched PCR (12,20) that enriches the mutant allele by the intermittent restriction digestion of the wild-type allele and a COLD-PCR (21) that enriches the mutant allele by means of the difference in melting temperatures between the mutant and wild-type alleles. The principle of the MEC-HRM assay is shown in Fig. 1A.

As the first step, conventional PCR was performed to amplify the target region (129 base pairs), including codon 790
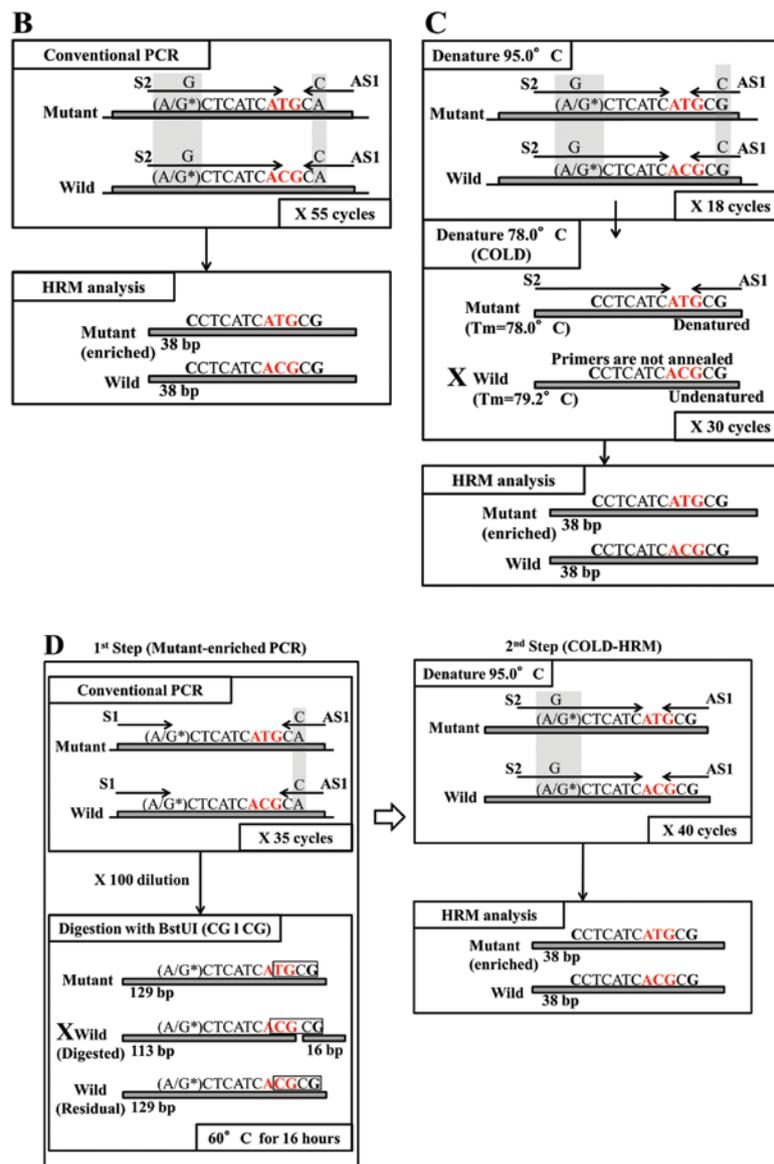
Figure 1. Continued. (B) Principle of standard HRM assay. The box indicates the real-time PCR step using the S2 primer (forward) and the AS1 primer (reverse). The real-time PCR conditions are as follows: an initial denaturation step at 95˚C for 10 min, and an amplification step for 55 cycles (95˚C for 15 sec, 60˚C for 60 sec). The final products are analyzed using HRM analysis. (C) Principle of COLD-HRM assay. The upper box indicates the first step of 18 cycles of real-time PCR for the amplification of the template product, and the lower box indicates the second step, which is a COLD-PCR step, for the selective amplification of the mutant allele. In the second step, the denaturing temperature (Td) is decreased to 78˚C and amplification is performed for 30 cycles. The final products are analyzed using HRM analysis. (D) Principle of mutant-enriched HRM assay. In the first step (left box), conventional PCR and digestion are performed, similar to that shown in the left box of (A). The second step (right box) is performed in the same manner as standard HRM (B) using the products from the first step. Forty cycles are used for the mutant-enriched HRM assay, instead of 55 cycles for the standard HRM assay.

of the *EGFR* gene, using the GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The forward primer sequence was 5'-ACTGACGTGCCTCTCCCTCC-3' (S1). The reverse primer sequence was 5'-CGAAGGGCATG AGCC*GC-3' (AS1), harboring one mismatched site (* T to C) to introduce the CGCG sequence into the amplicon of the wild-type. The final volume of the PCR mixture was 10 μl contained 100 ng of sample DNA, 150 nmol of deoxynucleotide triphosphate, 2 pmol of each primer, and 1 unit of HotStarTaq DNA Polymerase Plus (Qiagen). The PCR conditions were as follows: an initial denaturation step at 95˚C for 5 min, followed by 35 cycles of 94˚C for 20 sec, 60˚C for 30 sec, and 72˚C for 20 sec. Diluted PCR products (100-fold dilution with distilled water) were treated with the restriction enzyme *Bst*UI (New England BioLabs, Ipswich, MA, USA), which digests the wild-type allele but not the mutant allele, for 16 h at 60˚C, resulting in the enrichment of the mutant alleles, as previously reported by us (12).

After the first step, the second step (COLD-PCR, including melting curve analysis) was performed using the StepOnePlus™ real-time PCR system (Applied Biosystems). The forward primer sequence was 5'-CCTCCACCGTGCAC*CTCATC-3' (S2). Since one SNP site (rs1050171 A/G) exists in the S2 primer sequence, we designed a mismatched-base (* C) at the SNP position to diminish the influence of the SNP (28). We used the AS1 primer as a reverse primer. The final volume of the PCR mixture was 20 μl, containing 10 μl of MeltDoctor™ Master Mix (Applied Biosystems), 12 pmol of each primer and
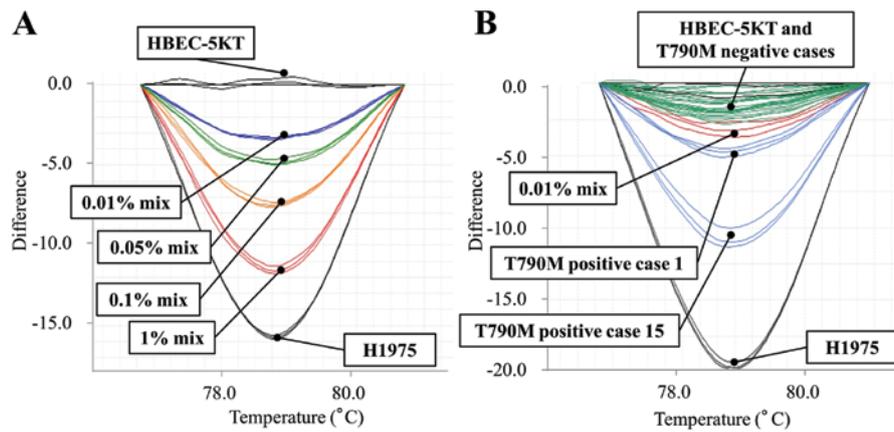
Figure 2. Sensitivity of MEC-HRM assay and representative clinical samples. (A) Difference plot showing the sensitivity of the MEC-HRM assay. We selected wild-type DNA (HBEC-5KT) for the standard of difference plots (horizontal line). Each percentage indicates the proportion of T790M-mutant DNA out of wild-type DNA. Consequently, the MEC-HRM assay was able to detect a T790M-mutant DNA (H1975) level of 0.01% among wild-type DNA (HBEC-5KT). (B) The red curves indicate a DNA mixture containing 0.01% H1975 DNA among HBEC-5KT DNA. The green curves indicate the clinical samples determined as T790M-negative, and the blue curves indicate the samples determined as T790M-positive. Case 1 contains a 0.01-0.1% proportion of T790M clones, and case 15 contains a 0.1-1% proportion of T790M clones.

1 μl of the first-step product. The real-time PCR conditions were as follows: an initial denaturation step at 95°C for 10 min, 18 cycles for the first round of standard amplification (95°C for 15 sec, 60°C for 60 sec), and 30 cycles for the second round of amplification to enrich the mutant-amplicons (78°C for 15 sec and 60°C for 60 sec). The denaturing temperature (Td) of the COLD-PCR step (78°C) was determined using a melting curve analysis of the standard HRM [the melting temperatures (Tm) of the mutant-type and wild-type amplicons were 78°C and 79.2°C, respectively] and an evaluation of the sensitivity of COLD-PCR from Td 79.2 to 78°C every 0.2°C. After the amplification step, a melting curve was generated and analyzed using HRM software ver. 3.0.1 (Applied Biosystems).

We also performed standard-HRM, COLD-HRM and mutant-enriched HRM assays. The principles of these three assays are provided in Fig. 1B-D. All the samples, including the standard DNA mixtures, were analyzed in triplicate in all the assays. The sensitivities of all four assays were determined using multiple DNA mixtures of T790M mutant and wild-type alleles (0.01, 0.05, 0.1, 1, 10, 20 or 50% of T790M mutant allele).

Statistical analysis. Chi-square tests or Fisher's exact tests were used to examine the differences in categorical factors across groups, as appropriate. The multivariate logistic regression model was used to identify clinicopathological factors that might independently predict the presence of T790M mutations.

After pulmonary resection, imaging studies were repeated every 3 months for at least 2 years and every 6 months thereafter for 3 years. After 5 years, medical examinations were repeated every year. The progression-free survival (PFS) was calculated from the date of surgery until confirmed disease recurrence or death. The overall survival (OS) was calculated from the date of surgery until the date of death or the last follow-up. The survival curve was calculated using the Kaplan-Meier method, and the difference between groups was compared using the log-rank test. Multivariate analyses were performed using the Cox proportional hazard model. All the data were analyzed using JMP, version 9.0.0 (SAS Institute Inc., Cary, NC, USA).

All the statistical tests were two-sided, and probability values (P) <0.05 were considered statistically significant.

Results

Sensitivity of each assay for the detection of the T790M mutation. The MEC-HRM assay was able to detect the T790M mutant allele in the sample with a mutant allele content of 0.01%. In other words, it detected the mutant allele in the presence of a 10,000-fold background of wild-type allele (Figs. 2 and 3). Meanwhile, the detection limits of standard HRM, COLD-HRM and mutant-enriched HRM assays were a mutant allele content of 10, 1 and 0.1%, respectively (Fig. 4). Based on these data, we investigated the T790M mutation in 146 clinical samples using the standard HRM using a DNA mixture with T790M content of 10% as a positive control, and the MEC-HRM assays using a DNA mixture with T790M content of 0.01% as a positive control. Clinical samples and positive controls were investigated in the same plate in triplicate. When all three signals from a clinical sample exceeded the maximal signal from the control, the sample was identified to be positive for the T790M mutation (Fig. 2).

EGFR mutations in the clinical samples. Drug-sensitive *EGFR* mutations were found in 54 (37%) of the 146 lung adenocarcinomas (26 exon 19 deletions and 28 L858R mutations). Drug-sensitive *EGFR* mutations were significantly associated with females (P<0.01) and never-smokers (P<0.01).

Although standard HRM did not detect any *EGFR* T790M mutations, *EGFR* T790M mutations were detected in 19 (13%) of the 146 lung adenocarcinomas using the MEC-HRM assay. Thus, these 19 patients were defined as harboring minor T790M mutations. We confirmed that these 19 tumor samples had a tumor cell composition of at least 20% by examining the proportion of tumor cells in the tissue using hematoxylin and eosin staining. Next, we determined the dosage of the T790M mutant allele in 19 mutant samples by reanalyzing these samples and comparing them with 10, 1, 0.1 and 0.01% standard T790M mutant DNA mixtures. We subcategorized the
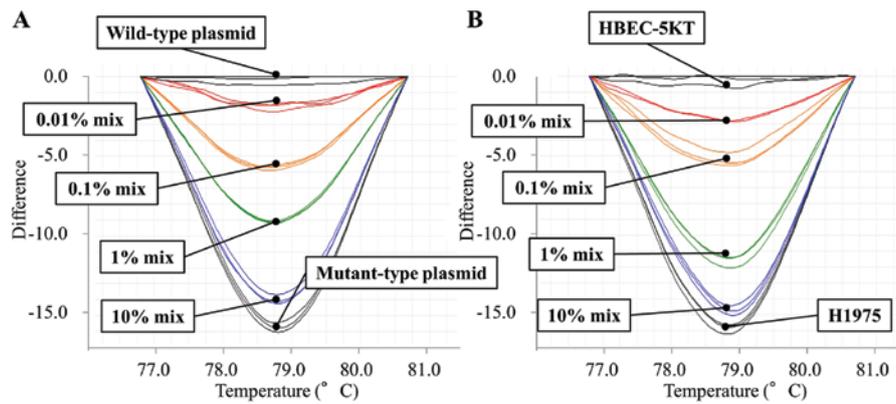
Figure 3. Comparison of sensitivities between plasmid and genomic DNA mixes. (A) The MEC-HRM assay was able to detect the T790M mutant allele at a level of 0.01% among wild-type alleles using the plasmid mixture. (B) The MEC-HRM assay showed similar sensitivities for the genomic DNA mixtures (H1975 and HBEC-5KT) and the plasmid mixtures.
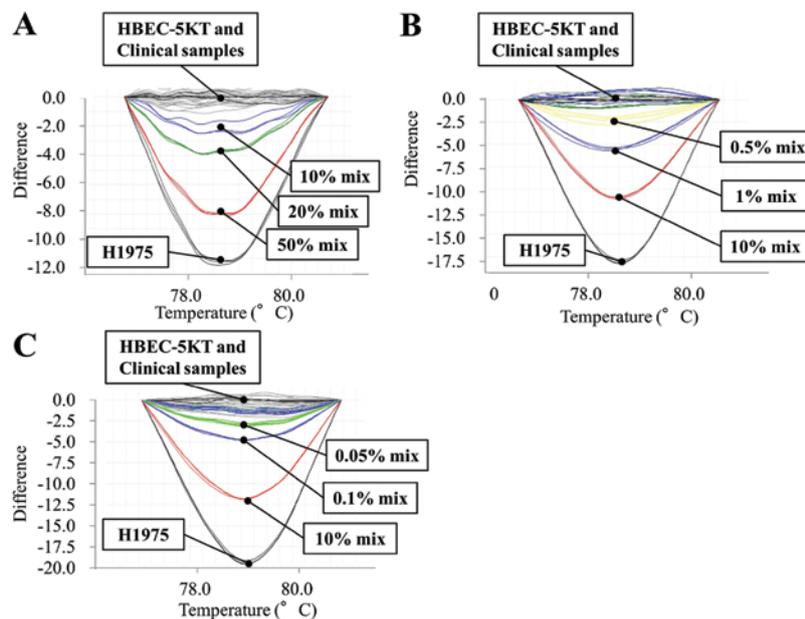

Figure 4. Sensitivities of standard HRM, COLD-HRM and mutant-enriched HRM assays. The difference plots for all three assays are shown. Wild-type DNA was used as a negative standard, and the percentages in each figure indicate the proportion of T790M-mutant DNA out of wild-type DNA. Consequently, the (A) standard HRM, (B) COLD-HRM and (C) mutant-enriched HRM assays were able to detect T790M-mutant DNA at levels of 10, 1 and 0.1% among wild-type DNA, respectively.

19 minor T790M mutations into groups with T790M mutant DNA levels corresponding to 0.01-0.1%, 0.1-1% and 1-10%. As shown in Table I, 17 of the 19 patients (84%) had a T790M mutant allele proportion of <0.1%.

The details of the 19 patients harboring minor T790M mutations and the relationships between a minor T790M mutational status and clinicopathological factors are shown in Tables I and II, respectively. Minor T790M mutation was not significantly associated with age, gender, pathological stage, smoking history or drug-sensitive *EGFR* mutations in univariate analyses. However, drug-sensitive *EGFR* mutations are considered to be associated with age, gender and smoking status (27,29,30); thus, a multivariate analysis was performed to identify independent factors associated with a minor T790M mutation. Consequently, the minor T790M mutation was found to be independently associated with drug-sensitive *EGFR* mutations (OR, 3.0; 95% CI, 1.0-9.0; P=0.04) (Table III).

Regarding the prognostic impact of the minor T790M mutation, it was not associated with either the OS or the PFS in our cohort (Table IV).

Discussion

In the present study, we determined the T790M mutational status using our newly developed, highly sensitive assay, the MEC-HRM assay. We previously developed a mutant-enriched PCR assay (sensitivity, 0.1%) and found that the T790M mutation was detected in 3.8% of NSCLC patients without a history of treatment with EGFR-TKIs (12). Su *et al* (16) found that the T790M mutation was present in 25.2% of EGFR-TKI-naive NSCLC patients using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (sensitivity, 1.5%), and Oh *et al* (13) found that the T790M mutation was present in 8.2% of EGFR-TKI-naive NSCLC

Table I. Details of the 19 T790M-positive patients.

Case	Age (years)	Gender	Smoking history	Pathological stage	Drug-sensitive *EGFR* mutation	Proportion of T790M clones
1	59	Male	Never-smoker	I	ex19 del.	0.01-0.1%
2	75	Male	Never-smoker	I	ex19 del.	0.01-0.1%
3	75	Female	Never-smoker	I	L858R	0.01-0.1%
4	75	Female	Never-smoker	I	L858R	0.01-0.1%
5	79	Female	Never-smoker	I	L858R	0.01-0.1%
6	60	Female	Never-smoker	I	WT	0.01-0.1%
7	60	Male	Never-smoker	III	WT	0.01-0.1%
8	66	Male	Smoker	I	ex19 del.	0.01-0.1%
9	57	Female	Smoker	I	ex19 del.	0.1-1%
10	76	Male	Smoker	I	ex19 del.	0.01-0.1%
11	82	Male	Smoker	I	L858R	0.01-0.1%
12	58	Male	Smoker	I	L858R	0.01-0.1%
13	56	Male	Smoker	I	WT	0.01-0.1%
14	32	Female	Smoker	I	WT	0.1-1%
15	59	Female	Smoker	I	WT	0.01-0.1%
16	78	Female	Smoker	I	WT	0.01-0.1%
17	68	Male	Smoker	I	WT	0.01-0.1%
18	74	Male	Smoker	I	WT	0.01-0.1%
19	84	Female	Smoker	II	L858R	1-10%

ex19 del., exon 19 deletion; WT, wild-type. Drug-sensitive *EGFR* mutations include detection of exon19 deletions and the L858R mutation.

Table II. Relationship between minor T790M mutations and clinicopathological factors.

Subsets (n=146)	T790M-positive (n=19) n, (%)	T790M-negative (n=127) n, (%)	P-value
Age (median; range) (68; 32-87 years)			
<68 (n=72)	9 (47.4)	63 (49.6)	0.9
≥68 (n=74)	10 (52.6)	64 (50.4)	
Gender (male vs. female)			
Male (n=71)	10 (52.6)	61 (48.0)	0.7
Female (n=75)	9 (47.4)	66 (52.0)	
Smoking history			
Smoker (n=74)	12 (63.2)	62 (48.8)	0.2
Never-smoker (n=72)	7 (36.8)	65 (51.2)	
Pathological stage			
I (n=103)	17 (89.5)	86 (67.7)	0.06
II (n=12)	1 (5.3)	11 (8.7)	(I vs. others)
III (n=21)	1 (5.3)	20 (15.7)	
IV[a] (n=10)	0 (0.0)	10 (7.9)	
Drug-sensitive *EGFR* mutation			
Mutant (n=54)	10 (52.6)	44 (34.6)	0.1
Wild (n=92)	9 (47.4)	83 (65.4)	

[a]Stage IV includes 4 recurrent patients. Drug-sensitive *EGFR* mutations include detection of exon19 deletions and the L858R mutation.

Table III. Multivariate analysis of the minor T790M mutation-related factors.

Variables	OR (95% CI)	P-value
Age (years)		
(<68 vs. ≥68)	1.0 (0.4-2.6)	1.0
Gender		
(Male vs. female)	0.6 (0.2-2.5)	0.5
Smoking history		
(Smoker vs. never-smoker)	3.8 (0.9-18)	0.08
Drug-sensitive *EGFR* mutation		
(Mutant vs. wild)	3.0 (1.0-9.0)	0.04

OR, odds ratio; CI, confidence interval. Drug-sensitive *EGFR* mutation include detection of exon19 deletions and the L858R mutation.

patients using peptide nucleic acid (PNA)-clamping PCR with a melting curve analysis (sensitivity, 0.01%). The present study revealed that the MEC-HRM assay could detect the T790M mutation in 13% of EGFR-TKI-naive adenocarcinomas. Of note, our standard HRM assay (10% sensitivity) did not detect any T790M-positive specimens, and we considered all the T790M mutations that were detected using the MEC-HRM assay to be minor mutations (2,17).

As novel findings, the minor T790M mutation was significantly associated with drug-sensitive *EGFR* mutations in a multivariate analysis. Previous reports have described that drug-sensitive *EGFR* mutations and the T790M mutation generally occur *in cis* (31-33), although the statistical relationship between minor T790M mutation and drug-sensitive *EGFR* mutations has never been revealed in clinical samples. Our present study is, to the best of our knowledge, the first report to confirm that the minor T790M mutation was significantly associated with drug-sensitive EGFR mutations in lung adeno-

carcinomas. To understand this situation, two issues should be discussed: i) the relationship between drug-sensitive *EGFR* mutations and the T790M mutation, and ii) the presence of the T790M mutation as a minor population. Regarding the first issue, while the reason for this association is unknown, lung cancers with germ-line *EGFR* mutations such as T790M (31), V843I (34), and R776H (35) are accompanied by additional *EGFR* mutations in the development of lung cancer, suggesting that *EGFR* mutations themselves cause genetic instability, thereby predisposing cells to additional mutations within the gene (34). The second issue can be explained by the following hypothesis. During the carcinogenic process of *EGFR*-mutant-related lung cancer, drug-sensitive *EGFR* mutations occur first in the progenitor cells of lung cancer, followed by the T790M mutation. At this stage, the tumor consists of a heterogeneous population of drug-sensitive *EGFR* mutant cells with or without the T790M mutation. Previous studies have reported that drug-sensitive *EGFR* mutant cells with an additional T790M mutation exhibit an indolent progression, indicating that the possession of the T790M mutation is a disadvantage for cell proliferation (36). This fact suggests that cancer cells with only drug-sensitive *EGFR* mutations may be dominant in tumors, in addition to those with T790M and drug-sensitive *EGFR* mutations as a minor population.

Our study also suggested that the T790M mutation tended to be associated with a smoking history in this study. Drug-sensitive *EGFR* mutations are known to be frequent in never-smokers with NSCLCs. However, drug-sensitive *EGFR* mutations themselves have not been reported to be associated with a never-smoking habit (30). Matsuo *et al* (30) indicated that *EGFR* mutations presumably occur in both smokers and never-smokers with a similar incidence, but other smoking-related mutations such as TP53 or KRAS mutations preferentially occur in smokers resulting in the enrichment of the prevalence of *EGFR* mutations in never-smokers. In fact, the average mutation frequency has been reported to be >10-fold higher in smokers than in never-smokers (37). Considering these observations, the *EGFR* mutation at codon

Table IV. Multivariate analysis for recurrence and mortality.

Variables	PFS		OS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (<68 vs. ≥68 years)	0.3 (0.1-0.6)	<0.01	0.3 (0.1-0.9)	0.02
Gender (Male vs. female)	0.7 (0.2-1.9)	0.4	1.0 (0.3-3.6)	0.9
Smoking history (Smoker vs. never-smoker)	2.3 (0.8-7.3)	0.1	2.1 (0.6-8.5)	0.3
Pathological stage (I vs. II-IV)	0.2 (0.09-0.4)	<0.01	0.2 (0.05-0.4)	<0.01
Drug-sensitive *EGFR* mutation (Mutant vs. wild)	1.0 (0.4-2.2)	1.0	0.5 (0.1-1.4)	0.2
T790M mutation (Mutant vs. wild)	0.5 (0.08-1.8)	0.3	1.7 (0.4-5.9)	0.4

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. Drug-sensitive EGFR mutations include detection of exon19 deletions and the L858R mutation.

790, unlike drug-sensitive *EGFR* mutations, might be susceptible to tobacco-related carcinogens.

In conclusion, we revealed an association between the minor T790M mutation and clinicopathological factors in surgically resected lung adenocarcinoma patients without a history of EGFR-TKI treatment using our novel, highly sensitive assay. Minor T790M mutations are independently associated with drug-sensitive *EGFR* mutations.

Acknowledgements

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